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DNA multi-CTL epitope vaccines for HIV and *Plasmodium falciparum*: immunogenicity in mice

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The potential of building multi-cytotoxic T lymphocyte (CTL) epitope antigens in combination with the nucleic acid immunization technology is explored for development of acquired immunodeficiency syndrome (AIDS) and malaria vaccines. A novel minimal vector pTH for direct gene transfer was constructed for efficient expression of vaccine antigens and used as a vehicle for human immunodeficiency virus (HIV)- and Plasmodium falciparum*-derived polyepitope genes. Two murine epitopes were included into these constructs to allow for testing of vaccine immunogenicity in small animals. The results showed that a single DNA injection generated CTL responses in all 15 vaccinated mice. The elicited CTL precursor frequencies were estimated in an interferon-γ (IFN-γ)-based ELISPOT assay and found to be an average of 300 (range 4–1346) peptide-responding cells per 10^6 splenocytes.* © *1998 Elsevier Science Ltd. All rights reserved*

Keywords: polyepitope; DNA; vaccine

Vaccination has provided the most effective prevention of infectious diseases since the introduction of water chlorination and pasteurization of milk. As new infections keep emerging and old ones that were thought to be controlled re-emerge, the list of vaccines required for children and adults grows. New ways of vaccination including vaccine combinations and simplified immunization schedules are therefore of a great value in prevention of infectious diseases.

An essential arm of the body's defense against many infections is the activity of $CD8^+$ cytotoxic T lymphocytes (CTL)[1]. Provided that this activity does not exacerbate the disease symptoms, a successful vaccine should expand the population of pathogen-specific CTL precursors. CTL recognize and kill cells displaying on their surface peptides derived from microbial proteins that are presented to CTL in association with major histocompatibility complex (MHC) class I molecules. One way to minimize the amount of protein administered during vaccination is to engineer artificial proteins consisting of CTL epitopes[2,3]. These epitopes can be derived from multiple proteins of one or several infectious agents and cover several known or predicted epitope variants which may arise from mutations escaping the CTL recognition. Unlike the use of whole proteins, this approach enables focusing of the immune response on important or highly conserved epitopes and the exclusion of protein domains that could induce infection-enhancing antibody or immunosuppression. An obstacle to epitope vaccine design is the polymorphism of MHC class I molecules that together with other proteins in the antigen processing pathway determine which peptides are displayed on the cell surface. Consequently, different individuals present different sets of peptides to CTL depending on their genetic make-up. It has been estimated that five MHC class I-restricted optimally selected epitopes could cover 80 to 90% of the Caucasian and Oriental populations and eight or nine would be required for total coverage of the general population irrespective of ethnic descent[4,5]. Thus, although the complexity of a broadly efficacious epitope-based vaccine may still be high, our increasing understanding of the immune system and the availability of rapid strategies for epitope determination have generated considerable interest in the potential applications of epitope-based vaccines to the prophylaxis and therapy of infectious diseases.

Direct DNA inoculation is a novel means of active specific immunization. It is very attractive for its simplicity, 'purity' and the flexibility with which complex vaccines can be built. Also, DNA vaccine preparations are significantly more stable than current protein-based vaccines and probably safer than viral-mediated gene transfers, particularly in a potentially immunocompromised host. 'Naked' DNA vaccination has been successfully used for *in vivo* induction of both humoral

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and cell-mediated immune responses, including MHC class I-restricted CTL[9–15]. Moreover, a successful development of a vaccination protocol in mice, whereby a single DNA injection dose provides sufficient expression of proteins to elicit strong immune responses, has generated a hope that similar efficacy could be achieved in primates including man.

Acquired immunodeficiency syndrome (AIDS) and malaria are two diseases which have in some areas a significant impact on public health and which are still waiting for an efficient vaccine. Although the correlates of protection remain elusive, the important role that CTL can play in the control of these infections has been supported for both the human immunodeficiency virus (HIV), the causative agent of AIDS, and *Plasmodium falciparum*, the parasite causing severe malaria[16–25]. Induction of MHC class I-restricted CTL responses in the absence of antibody in high risk uninfected individuals can be one way to evaluate the role of CTL in preventing infection or, in the case of HIV, attenuating it to decrease virus load and delay the onset of AIDS. For this approach, vaccines efficiently eliciting CTL are required.

In this work, the potential of building polyepitope vaccines together with the power of DNA immunization is explored for the development of CTL vaccines against AIDS and malaria, and for a vaccine combination approach in general. Initially, two polyepitope genes were constructed. The first gene, designated H, was derived from human and simian (SIV) immunodeficiency virus sequences and contained mostly human, but also macaque and murine CTL epitopes. The second gene, designated M, was originally assembled for a virus-like Ty particle-based vaccine (S.C. Gilbert *et al.*, manuscript submitted for publication) from known human CTL epitopes of the liver-stage proteins of *Plasmodium falciparum* together with a murine epitope derived from *Plasmodium berghei*. The H and M genes were inserted into a novel DNA vaccine vector pTH either on their own or as one combined multi-epitope gene called HM. As the first stage of testing of these recombinant vaccines prior to commencing primate trials, the vectors were inoculated into mice and the immunogenicities of the two murine epitopes were determined.

MATERIALS AND METHODS

DNA immunization vectors

The backbone of the DNA immunization vectors was derived from plasmid pRc/CMV (Invitrogen). Firstly, the plasmid was cut with *Bam*HI (there are 3 *Bam*HI sites in pRc/CMV), the fragments carrying ColE1, β-lactamase and CMV promoter, and bovine growth hormone polyadenylation site were gel-purified and re-ligated giving plasmid pCMVBam. This plasmid was then partially cut with *Bam*HI, the single-cut DNA was recovered from a gel, the staggered ends were filled in using the Klenow polymerase and re-ligated. The plasmid with a unique *Bam*HI site in the polylinker was designated pCMV. The enhancer/promoter region of pCMV was then excised using *Mlu*I and *Hin*dIII restriction endonucleases and a enhancer/immediate early promoter/intron A region of the human cytomegalovirus (CMV) was ligated between the two sites resulting in a plasmid designated pTH. The pTH vector was used as a vaccine vehicle for genes in direct gene transfers.

Construction of multi-epitope DNA vectors

The polyepitope gene H containing the HIV- and SIV-derived sequences was constructed using six partially overlapping oligonucleotides Sup1 to Sup6 (R&D Systems) in a polymerase chain-reaction (PCR) assembly. First, 3 separate PCRs were carried out employing Sup1 and Sup2, Sup3 and Sup4, and Sup5 and Sup6 primers. In the second step, Sup1-2 and Sup3-4 PCR products were added into the reaction mixture as template together with primers Sup1 and Sup4. Finally, the full-length gene H was amplified using PCR products of Sup1-4 and Sup5-6 as templates and primers Sup1 and Sup6. Multiple nucleotide sequence errors in the final PCR product were subsequently corrected using site-directed mutagenesis[26]. The correct gene H was inserted between the *Hin*dIII and *Pst*I sites of plasmid pTH and the resulting pTH.H plasmid was used for immunizations.

To construct the pTH.M plasmid derived from *Plasmodium* genes, cassettes CAB containing total of nine CTL epitopes (S.C. Gilbert *et al.*, manuscript submitted for publication) were modified using PCR. A *Bam*HI site was put at the 5′ end followed by a Kozak sequence and ATG, and the 3′ *Bam*HI site was converted to a *Bgl*II site. Cassettes D and H were then added to the 3′ end of CAB and the CABDH gene was ligated into the pTH.H plasmid, which was digested with *Bgl*II (partial) and *Bam*HI to remove the HIV epitopes but leave the mAb Pk tag in frame.

Plasmid pTH.HM coding for both the HIV and *Plasmodium* epitopes was generated by inserting the *Bgl*II-*Bam*HI CAB cassette fragment into the *Bam*HI site preceding the Pk tag in pTH.H.

Cells

293T and COS-1 cells were maintained in Dulbecco's Modified Eagles Medium (Gibco) supplemented with 10% fetal calf serum (FCS; Gibco) and penicillin/streptomycin (P/S). P815 mastocytoma cell line was grown in RPMI 1640 (Gibco) supplemented with 10% FCS and P/S. All cells were cultured in humidified incubators in 5% CO_2 at 37°C.

Transient transfection of 293T and COS-1 cells

293T cells were grown in 15-mm tissue culture plates to approximately 70% confluency and transfected using the calcium phosphate precipitation method[27]. The cells were harvested after 3 days, resuspended in disruption buffer [5% glycerol, 50 mM Tris-HCl, pH 7, 2% sodium dodecylsulfate (SDS), 5% 2-mercaptoethanol, 0.25% bromophenol blue] and stored at −70°C until required.

Transfection of COS-1 cells was performed using the DEAE–dextran–chloroquine method[28]. Briefly, 2.5×10^5 COS-1 cells were grown overnight on coverslips in 6-well tissue culture plates. The following day cells were transfected with 5 μg DNA well^{-1} and stained for the Pk tag expression 48 h later.

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Antibodies

Monoclonal antibody SV5-P-k specific for the Pk epitope was described previously[29] (Serotech). R4 (ATCC) and biotin-conjugated XMG1.2 (Pharmigen) mAbs were used in the ELISPOT assay for detection of murine IFN-γ. FITC-conjugated goat anti-mouse antibodies (Sigma) were used in the fluorescent microscopy.

SDS–PAGE and Western blot analysis

Cell lysates suspended in disruption buffer were boiled for 2 min, centrifuged and individual polypeptides were separated on SDS–polyacrylamide gels cross-linked with 15% (*N,N*-diallyltartardiamide (DATD) using thin (0.75 mm) mini-slab gels of the Bio-Rad electrophoresis system. Separated polypeptides were transferred onto a nylon filter (Amersham International) using a semi-dry gel electroblotter (LKB). The filters were blocked with phosphate-buffered saline (PBS) plus 20% (*w/v*) skimmed milk and incubated with SV5-P-k mAb in PBS plus 5% (*w/v*) skimmed milk. Bound antibodies were detected using horseradish peroxidase (HRP)-conjugated protein A (Amersham International) in PBS plus 5% (*w/v*) skimmed milk followed by enhanced chemiluminescence (ECL; Amersham International).

Fluorescence microscopy

Transfected COS-1 cells were fixed in 2% paraformaldehyde in PBS and permeabilized using 0.2% Triton X-100 in PBS. The expressed proteins were detected using the SV5-P-k mAb (Serotech) followed by FITC-conjugated goat anti-mouse antibodies (Sigma) and fluorescent microscopy.

DNA Immunization

Plasmid DNA used for immunization was prepared using the Qiagen Megaprep Columns. Balb/c mice were injected into skeletal muscles as described previously[80]. Briefly, hair was removed from both lower hind legs and the calf muscles were injected with 50 μl 10^{-5} M cardiotoxin, a 60-amino acid-residue peptide isolated from *Naja nigricollis* venom (Latoxan) resuspended in 0.9% NaCl to cause muscle regeneration. Six days later, a total of 100 μg plasmid DNA in endotoxin-free PBS (Sigma) was injected into the same muscles. Mice were sacrificed 10 days after the vaccination and the immunogenicity of the vaccine was assessed.

CTL cultures

Spleens were removed and pressed individually through a cell strainer (Falcon) using a 2-ml syringe rubber plunger. The splenocytes were washed twice, suspended in 10 ml Lymphocyte medium [RPMI 1640 supplemented with 10% FCS, P/S, 20 mM HEPES and 15 mM β-mercaptoethanol] and incubated with 2 μg ml^{-1} peptide in a humidified incubator in 5% CO_2 at 37°C for 5 days.

Target cells and standard ^{51}Cr-release assay

The effector cells were double-diluted in U-bottom wells (96-well plate; Costar) to yield from 100:1 to 3:1 effector-to-target ratios. Five thousand ^{51}Cr-labeled P815 cells in a medium containing 10^{-5} M peptide were then added to the effectors and incubated at 37°C for 4 h. Percent specific lysis was calculated as [(sample release − spontaneous release)/(total release − spontaneous release)] × 100. The spontaneous release was lower than 20% of the total c.p.m.

ELISPOT assay

The ELISPOT assay for detection of IFN-γ-releasing cells upon peptide stimulation was described previously[31]. Briefly, 96-well nitrocellulose-backed plates (MAHA S45, Millipore) were coated with 100 μl of 15 μg ml^{-1} of murine IFN-γ-specific mAb R4 (ATCC) overnight at 4°C, washed 6× with PBS and blocked using a medium supplemented with 10% FCS at room temperature for 1 h. Two dilutions of 5×10^5 and 2.5×10^5 of freshly isolated splenocytes and 2 μg ml^{-1} of a specific peptide were then added into the wells and incubated at 37°C, 5% CO_2 overnight. 5×10^5 cells without peptide were incubated as a negative control. The cells were washed 3× with PBS then 1 μg ml^{-1} secondary biotin-conjugated antibody XMG1.2 (Pharmigen) was added and reacted at room temperature for 3 h. The wells were washed 6× with PBS and AP-conjugated streptavidin (Sigma) was added at 1:1000 dilution for 1 h. The wells were again washed 6× with PBS and the spots were developed by adding peroxidase substrate 3,3′-diaminobenzidinetetrahydrochloride dihydrate (DAB; Sigma). After 15 min the wells were washed with tap water, dried and the spots were counted under a dissection microscope.

RESULTS

Construction of DNA vaccines

A novel vector for a direct gene transfer was constructed from parental plasmid pRc/CMV (*Figure 1*). Firstly, to minimize the size of the vaccine DNA, the neomycin resistance gene and f1 origin for single-stranded DNA replication were removed. The pRc/CMV enhancer/promoter region was then substituted with a more expression efficient enhancer/promoter/intron cassette containing the human CMV immediate early promoter. The resulting vector was designated pTH and used throughout this work. It is approximately 5.5 kbp in size and contains, in addition to the promoter cassette, a polyadenylation signal of the bovine growth hormone gene, β-lactamase gene and ColE1 origin of replication assuring a high plasmid copy number in bacteria.

The CTL polyepitope gene H derived from the HIV and SIV sequences was PCR assembled *in vitro* using six partially overlapping synthetic oligonucleotides (*Figure 2*). The open reading frame (ORF) is preceded by a Kozak 12-nucleotide consensus sequence to favor the initiation of translation from the first methionine codon of the recombinant protein gene[32]. A sequence coding for nine-amino acid-residue tag Pk recognized by monoclonal antibody (mAb) SV5-P-k[29] was coupled to the 3′-end of the polyepitope gene. A site for restriction endonuclease *Bam*HI was generated between the CTL polyepitope and Pk tag coding sequences to facilitate future addition of CTL epitopes.

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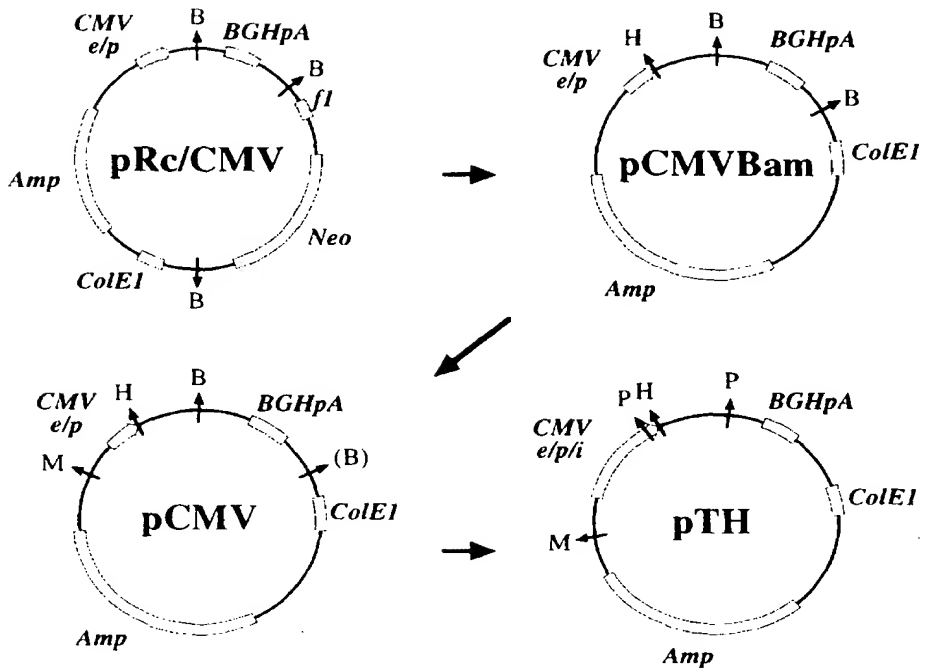

Figure 1 Construction of vector pTH for direct gene transfer. The pTH DNA vaccine vector was derived from plasmid pRc/CMV. First, the neomycin-resistance gene and M13 f1 origin of replication were removed and the *Bam*HI site in the plasmid polylinker was made unique. The pRc/CMV promoter was then substituted with an enhancer/promoter/intron region of the human cytomegalovirus. CMVe/p–CMV enhancer/promoter region; CMVe/p/i–CMV enhancer/promoter/intron region; BGHpA–bovine growth hormone polyadenylation signal; Amp–β-lactamase gene; ColE1–origin of plasmid replication; Neo–neomycin resistance gene; f1–M13 origin of replication; B–*Bam*HI; H–*Hin*dIII; M–*Mlu*I; P–*Pst*I

The H gene contains 1 murine, human (restricted by 12 different HLA molecules) and 3 rhesus macaque CTL epitopes (*Figure 3*). All the HIV-1 human epitopes are derived from the common European/North American clade B viruses and the corresponding CTL were detected in HIV-infected individuals. The restricting HLA molecules should enable over 90% of the above population to mount CTL responses. Where possible, advantage was taken of overlapping CTL epitopes to minimize the protein size, which totals 134 amino acid residues. Plasmid pTH.H was generated by inserting the H gene between the *Hin*dIII and *Pst*I sites of the pTH polylinker.

Construction of the *Plasmodium*-derived CTL polyepitope genes has been described elsewhere (S.C. Gilbert *et al.*, manuscript submitted for publication). Briefly, genes coding for cassettes of three epitopes and flanked by *Bgl*II and *Bam*HI sites at their respective 5′ and 3′ ends were assembled using synthetic oligonucleotides. Multi-cassette genes were then built by sequential insertions of individual 3-epitope cassettes into the 3′ *Bam*HI site, therefore each group of three epitopes in the protein is separated by GS amino acid residues. The M gene product contains 1 murine epitope of *P. berghei* and 11 human epitopes of *P. falciparum*, which are followed by 3 promiscuous helper epitopes derived from *P. falciparum* circumsporozoite protein, *Mycobacterium tuberculosis* 38 kDa antigen and tetanus toxin (*Figure 3*). The M protein contains 173 amino acid residues. To generate plasmid pTH.M, the Kozak and *Plasmodium* CTL/helper epitope sequences were inserted between the *Bgl*II and *Bam*HI sites of pTH.H, thus replacing the H polyepitope gene with the M gene. Finally, plasmid pTH.HM expressing both the HIV and 'malaria' epitopes (221 amino acid residues) was constructed by cloning of the three proximal cassettes of the M gene into the unique *Bam*HI site of pTH.H (*Figure 3*).

Expression and subcellular localization of multi-epitope proteins

Expression of the recombinant proteins from the novel pTH vector was confirmed *in vitro* prior to testing their immunogenicities in experimental animals. A monolayer of 293T cells was transiently transfected with vectors pTH.H, pTH.M or pTH.HM and cultured for 2 days. The cells were then lyzed, the soluble fractions were separated through an SDS–polyacrylamide gel and Western blotted, and the recombinant proteins were detected using the Pk tag-specific mAb followed by HRP-conjugated protein A and enhanced chemiluminescence (*Figure 4*). The full-size H, M and HM polyepitopes migrated according to their predicted respective relative molecular masses of 15.3 kDa, 18.8 kDa and 24.9 kDa. As expected for these artificially created proteins, some partially degraded faster-migrating species were observed.

Subcellular localization of the H, M, and HM gene products in COS-1 cells was visualized by immunofluorescence. COS-1 cells were transiently transfected with plasmid DNAs, the transfected cells were fixed, their membranes were permeabilized and the SV5-P-k mAb followed by anti-murine FITC-conjugated antibodies were used to detect the expressed recombi-

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nant proteins. All three polypeptides appeared to accumulate in similar vacuolar structures in the cytoplasm. An example of the immunofluorescence and corresponding phase-contrast images is shown for the pTH.HM-transfected COS-1 cells (*Figure 5*). Together, these results demonstrated efficient expression of the polyepitope genes from the pTH vector in eukaryotic cells.

Induction of CTL by DNA vaccination

To be able to test the immunogenicity of the DNA vaccines in a small-animal model, two CTL epitopes presented by murine MHC molecules were included into the vaccine antigens. The first epitope RGPGRAFVTI is a part of the H gene. It is presented by the H-2D^d molecule and was derived from the V3 region of the HIV-1 envelope glycoprotein[33]. The second well-defined H-2K^d-restricted CTL epitope SYIPSAEKI is included in the M gene and originates from the circumsporozoite protein of *P. berghei*[23].

In the first experiment, a group of mice was vaccinated using the pTH.HM vector and the *in vivo* priming of CTL was assessed in a standard ^{51}Cr-release CTL assay. Immune splenocytes were isolated 10 days post-injection and their bulk cultures were peptide-restimulated for 5 days. The assay showed that a single vaccine dose elicited CTL capable of lyzing P815 cells sensitized with both HIV and *Plasmodium* peptides (*Figure 6*). At the highest effector to target ratio of 100:1, the net specific lyses were 28, 38 and 22% for the HIV epitope and 21, 35 and 13% for the *Plasmodium* epitope of total c.p.m. released by a detergent treatment of target cells. Splenocytes from mice immunized with an irrelevant DNA and restimulated for 5 days with specific peptides did not show any peptide-specific CTL activity (data not shown).

In the second experiment, mice were vaccinated with either plasmid pTH.H, pTH.M or pTH.HM. Again, a single DNA vaccine dose elicited CTL responses in all immunized animals (*Table 1*). The observed specific lyzes ranged from 10% (animal m4) to 51% (animal h3) of the total ^{51}Cr release. This time, the frequencies of *ex vivo* peptide-specific splenocytes were estimated using an ELISPOT assay for detection of IFN-γ-producing cells[31]. It was shown previously that the ELISPOT responses are dependent on the presence of short MHC class I-restricted peptides and

(A)

Sup1 5'-CTCGAGAAGCTTAGATCTCCCGCCGCCACCATGGAAAGATACC
TAAAGGATCAACAGCTCCTGGAGATCACTCCAATTGGcttggcccc
acctatc-3'

Sup2 5'-gtacatgcggactatcTTGTTCAAACCCAGTATGATCCATCTT
TTGTAAATCTCTCCTACTGGgataggtggggccaag-3'

Sup3 5'-gatagtccgcatgtacAATCTAACGATGAAGTGTCGTATCCAG
AGAGGACCAGggagagcatttgttac-3'

Sup4 5'-gccttgtaggtcattgGTCTTAAAGGTACCTGAGGTCTTAACA
TCTGGTTAATGTCATAGGGTGTCTTTCCTATTgtaacaaatgctct
cc-3'

Sup5 5'-caatgacctacaaggcAGTCGATCTTAGCCACTTCTTAAAAGA
AAAGGGTGGACTGATTCTAAAAGAACCAGTACATggagtgtaccat
ccag-3'

Sup6 5'-GAATTCCTGCAGTCAGTCCAATCCCAGCAAAGGGTTTGGAATG
GATCCTAGGTCATCCATGTATTGATAGATAACTATGTctggatggt
acactcc-3'

(B)

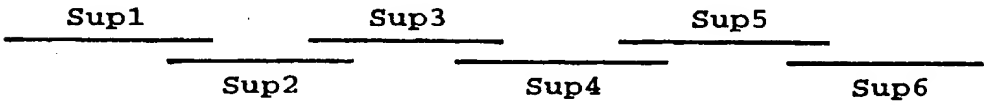

Figure 2 Synthesis of the HIV/SIV polyepitope gene. Panel A gives the nucleotide sequences of the set of six primers used for the PCR assembly of gene H. The complementary regions are shown in lower case. Panel B schematically depicts the gene assembly

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the IFN-γ is almost solely produced by $CD8^+$ T cells (A. Lalvani *et al.*, manuscript in preparation). An example of the IFN-γ 'footprints' obtained in the ELISPOT assay is shown for mouse h1 vaccinated with plasmid pTH.H (*Figure 7*). In that instance, an average of 673 positive signals per 5×10^5 freshly isolated splenocytes was counted. On average 300 (range 4–1346) responding cells per 10^6 splenocytes were detected (*Table 2*) and the percent specific lysis at the effector-to-target ratio of 100:1 after a 5-day peptide restimulation directly correlated with a logarithmic plot of the responder frequencies with the correlation coefficient $R^2 = 0.6$ (*Figure 8*). Peptides failed to stimulate IFN-γ production in splenocytes from unprimed animals (data not shown).

At the time of sacrifice, all DNA-vaccinated animals appeared normal and active suggesting that expression of the novel recombinant proteins in skeletal muscles did not cause any obvious adverse reactions or discomfort.

DISCUSSION

In the present report, a novel minimal DNA vector pTH for direct gene transfer was developed (*Figure 1*), which provided an efficient expression of the vaccine genes in eukaryotic cells. The gene products were easily detectable via a C-terminal tag Pk recognized by a mAb (*Figs 4 and 5*) and, after a single vaccination dose, generated CTL responses in 15 out of 15 vaccinated animals (*Figure 6* and *Table 1*). In animals immunized with a combined HIV/*Plasmodium* polyepitope vaccine, equal CTL responses were elicited against two present epitopes (*Figure 6* and *Table 1*).

The various pitfalls of antigen processing into peptides, the peptide transport, association with MHC molecules and presentation of the MHC–peptide complexes on the cell surface continue to be an area of active research. In particular, the negative influence of adjacent protein sequences on the generation of epitopes was demonstrated in some special situations[34], while other epitopes flanked by 'unnatural' amino acid residues were efficiently excised[35–37]. Epitopes from within recombinant polyepitope proteins were processed and presented to $CD8^+$ CTL, and both *in vitro* sensitized target cells[2] and *in vivo* primed CTL responses[3,38]. Here, further examples were provided where murine epitopes within H, M and HM multiepitope proteins were generated in vivo. For the HM protein, the two epitopes restricted by different $H\text{-}2^d$ molecules induced CTL responses with similar efficiencies (*Figure 6* and *Table 1*). Furthermore, preliminary results suggested that three rhesus macaque epitopes

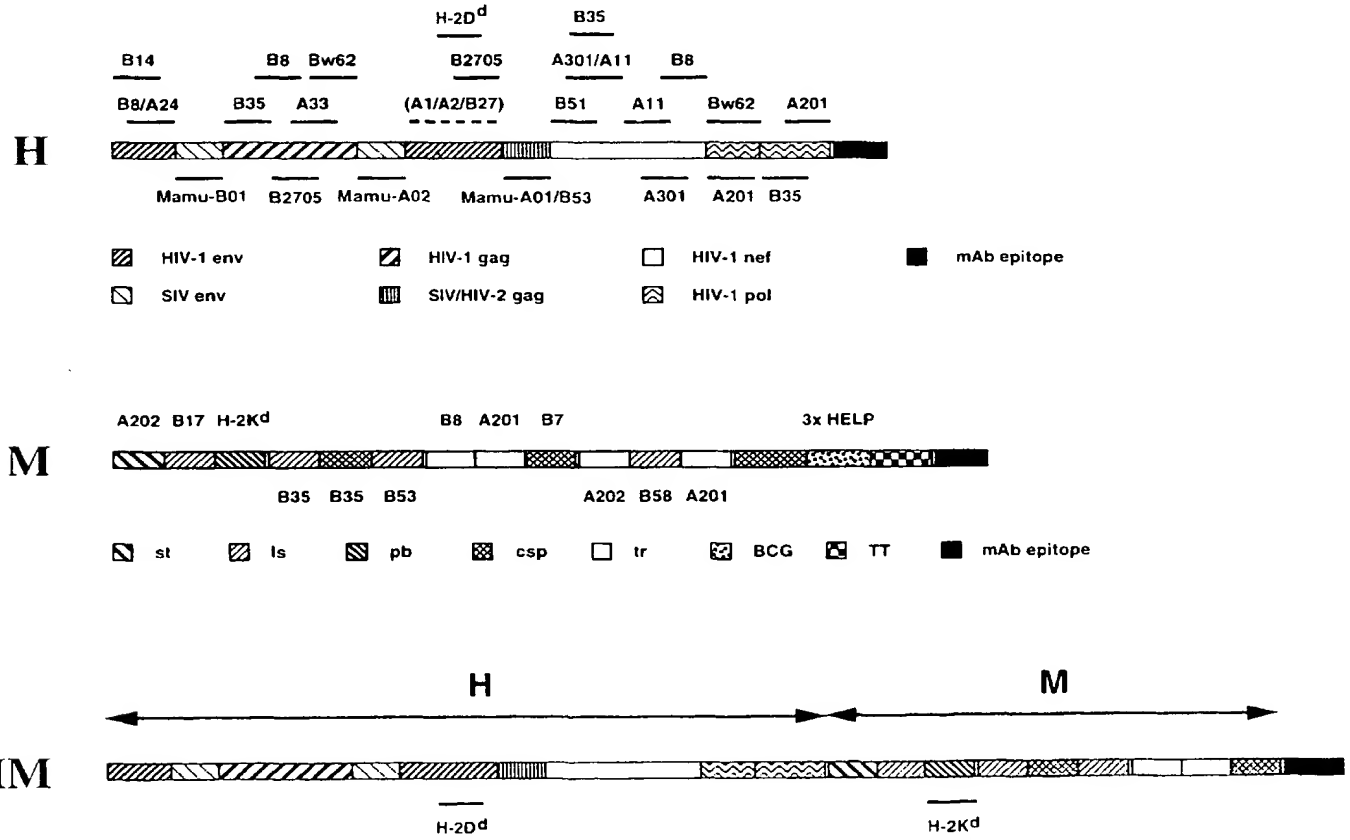

Figure 3 Schematic outline of the H, M and HM proteins. The bar patterns on the schematic representations of the polyepitope proteins indicate the origin of the sequences. The positions of individual epitopes and their MHC restrictions are depicted above and below the proteins. pb is the only epitope derived from the circumsporozoite protein of *P. berghei*. All other epitopes in the M protein originate from proteins of *P. falciparum*: cs-circumsporozoite protein, st–STARP (sporozoite threonine- and asparagine-rich protein), ls–LSA-1 (liver stage antigen 1) and tr–TRAP (thrombospondin-related adhesive protein). BCG–38 kDa protein of *M. tuberculosis*: TT–tetanus toxin (S.C. Gilbert et al., manuscript submitted for publication)

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of the H polypeptide elicited in vivo CTL responses (T. Hanke, unpublished data) and several human epitopes in H and HM proteins sensitized target cells to lysis by CTL lines or clones (T. Hanke *et al.*, manuscript submitted for publication). Thus, processing of individual epitopes from polyepitope antigens is in most cases successful.

Approximate frequencies of CTL precursors specific for the vaccine-encoded antigens were determined in an ELISPOT assay detecting IFN-γ-producing cells upon H-2D^d- and K^d-restricted peptide stimulation. The frequencies of stimulated cells after DNA priming (*Table 2*) were comparable to the ELISPOT frequencies detected following immunization with a recombinant vaccinia virus expressing the *P. yoelii* circumsporozoite protein[31] and correlated with the levels of cytotoxic activity (*Figure 8*). Although a component of CD4$^+$ Th1 responding cells cannot be excluded, it was shown in other experiments that the responding T cells in assays using MHC class I-restricted peptides are almost entirely CD8$^+$ (A. Lalvani *et al.*, manuscript in preparation). Thus, this simple, quick and sensitive method might in future substitute for the more conventional ^{51}Cr-release assay and overcome the need for MHC-matched target cells, which would significantly facilitate the testing of vaccines in primate models where the establishment of autologous B lymphoblastoid cell lines is often laborious and difficult.

Finally, the C-terminal Pk tag proved to be useful for confirming the presence of a full-length ORF of the *in vitro*-assembled genes and determining the subcellular localization of the gene products. To date, the Pk tag has been shown to retain its antigenicity when attached to either end of to up to 30 different proteins[29,39,40] (also R.E. Randall, University of

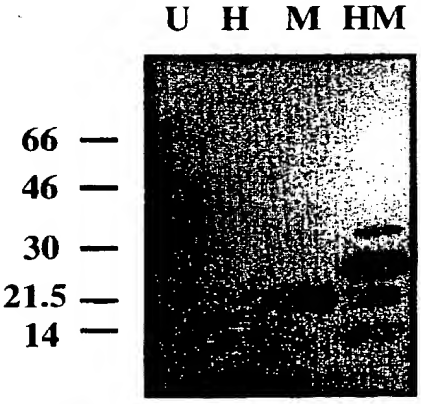

Figure 4 Expression of polyepitope proteins in transfected cells. 293T cells were transiently transfected with individual DNA vectors and the expression of the corresponding gene products was assessed in a Western blot analysis using the C-terminal Pk tag and tag-specific mAb followed by HRP-conjugated protein A and ECL. Lane U shows untransfected 293T cells and lanes H, M and HM are polypeptides detected in cells transfected with pTH.H, pTH.M and pTH.HM plasmids, respectively. The positions of relative molecular mass markers are indicated on the side

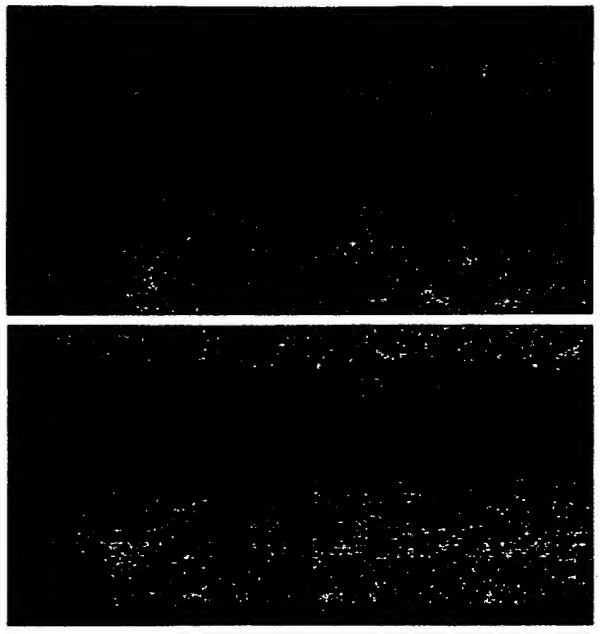

Figure 5 Subcellular localization of polyepitope proteins. COS-1 cells were transiently transfected with plasmid pTH.HM, fixed, permeabilized and the recombinant proteins were detected using SV5-P-k mAb followed by anti-mouse FITC-conjugated polyclonal antibody. The top image shows the subcellular localization of the HM polypeptide visualized under a fluorescence microscope. Both transformed (left) and untransformed (right) cells are shown. In the bottom half of the figure, the same two cells are shown in a phase-contrast image

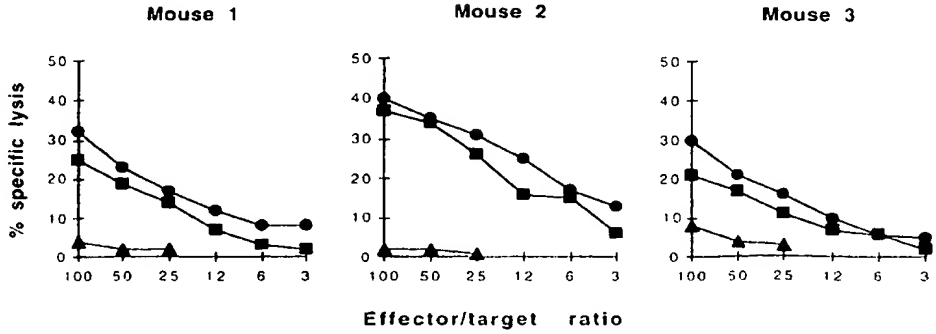

Figure 6 Cytotoxic T lymphocyte activities in mice immunized with the pTH.HM DNA vaccine. Mice were vaccinated intramuscularly with a single dose of 100 μg plasmid DNA, sacrificed 10 days later and the splenocytes were *in vitro* peptide-restimulated for 5 days. The cytotoxic activity of these bulk cultures were tested in a 4-h ^{51}Cr-release assay on P815 cells alone (triangles), or P815 cells in the presence of 10^{-7} M HIV peptide (circles) or *Plasmodium* peptide (squares)

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Table 1 Induction of CTL responses by i.m. inoculation of pTH.H, pTH.M and pTH.HM DNA vaccines[a]

Mouse	Vaccine	P815 cells plus:[b]		
		HIV peptide	*Plasmodium* peptide	No peptide
h1	pTH.H	41, 30, 27[c]	nd[d]	−1, 0, 3
h2	pTH.H	34, 19, 12	nd	0, 0, 0
h3	pTH.H	50, 51, 29	nd	1, 2, 0
h4	pTH.H	13, 9, 4	nd	2, 3, 2
m1	pTH.M	nd	21, 19, 16	3, 2, 1
m2	pTH.M	nd	19, 13, 7	8, 9, 6
m3	pTH.M	nd	28, 16, 11	3, 2, 3
m4	pTH.M	nd	10, 7, 3	2, 2, 1
hm1	pTH.HM	23, 20, 9	17, 9, 8	2, 1, 1
hm2	pTH.HM	29, 29, 20	25, 19, 15	3, 1, 1
hm3	pTH.HM	16, 14, 6	15, 12, 5	3, 0, 1
hm4	pTH.HM	34, 30, 24	34, 25, 20	2, 2, 1

[a]Mice were injected once with a total of 100 μg plasmid DNA into cardiotoxin-pretreated calf muscles.
[b]Peptides were added into the assay wells at concentration of 10^{-7} M.
[c]The cytotoxic activity is shown as percent specific lysis at effector to target ratios of 100:1, 50:1 and 25:1.
[d]Not done.

St.Andrews, personal communication) and to be in those positions only marginally immunogenic[21].

Fully functional polyepitope genes expressed from recombinant vaccinia viruses were reported previously[2,3,55] and the construction of polyepitope 'string-of-beads' vaccines for combining epitopes from multiple pathogens or escape mutants are recognized concepts in the vaccine field. However, the AIDS and malaria DNA vaccines constructed in the course of this work are unique in:

(1) the large number of CTL epitopes they contain, including their flexibility for easy future epitope addition;
(2) the partial use of overlapping epitopes, which goes beyond the 'string of beads' in reducing the amount of protein produced;
(3) they are to our knowledge the first DNA-based multi-epitope vaccines; and
(4) although designed for use in humans, they bear murine and rhesus macaque epitopes.

These epitopes were included so that the same vaccines can be used to test the immunogenicity and optimal vaccination doses, routes and regimes in experimental animals. The information gained especially in macaques, which are closer in size and immune responsiveness to humans than small animals, will allow effective planning of trials in humans.

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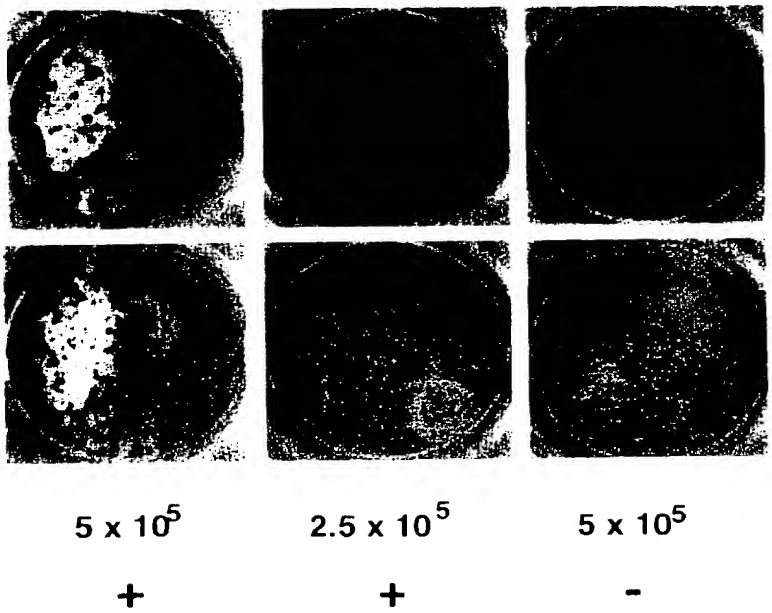

Figure 7 ELISPOT assay for detection of IFN-γ-producing cells. An example of duplicate ELISPOT signals obtained after HIV peptide stimulation of 5×10^5 and 2.5×10^5 splenocytes removed from h1 mouse of the 2nd vaccination experiment is shown. Each dot represents a 'footprint' of a IFN-γ-producing cell. $5 \cdot 10^5$ splenocytes incubated in the absence of peptide served as a negative control

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Table 2 ELISPOT-determined frequencies of IFN-γ-producing cells upon stimulation with CTL epitope peptides in freshly isolated spleens[a]

Mouse	Vaccine	Stimulation	
		HIV peptide	*Plasmodium* peptide
h1	pTH.H	1,346	nd[b]
h2	pTH.H	1,170	nd
h3	pTH.H	390	nd
h4	pTH.H	10	nd
m1	pTH.M	nd	76
m2	pTH.M	nd	4
m3	pTH.M	nd	22
m4	pTH.M	nd	6
hm1	pTH.HM	18	366
hm2	pTH.HM	nd	nd
hm3	pTH.HM	12	4
hm4	pTH.HM	397	367

[a]The listed frequencies were determined from wells where 5×10^5 cells were plated and are shown as a number of responding cells per 10^6 splenocytes.
[b]Not done.

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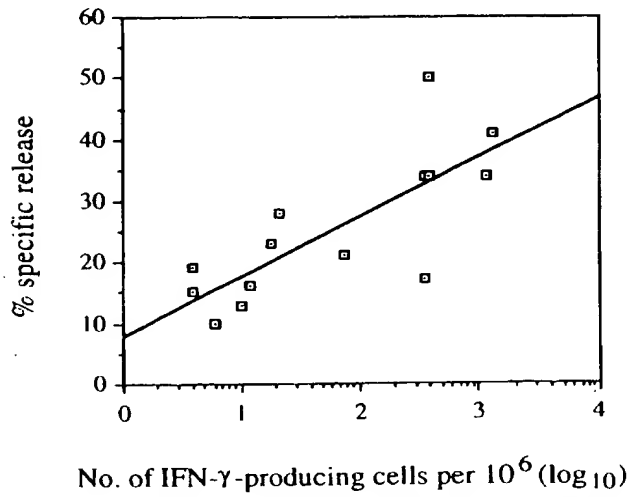

Figure 8 Correlation of cytotoxic activity and frequency of IFN-γ-producing cells from *Table 1* and *Table 2*. The correlation coefficient was $R^2 = 0.6$

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